

Supporting Information

Vollmers et al. 10.1073/pnas.0909591106

SI Text

Animals. All animal experiments and procedures were conducted following protocols approved by the Institutional Animal Care and Use Committee of the Salk Institute. Male wild-type C57BL/6J mice (8- to 12-week-old) were purchased from Jackson Laboratories. *cry1*^{-/-}; *cry2*^{-/-} mice were a kind gift from Dr. Aziz Sancar's laboratory. After arrival in Salk, the *cry1*^{-/-}; *cry2*^{-/-} mice were backcrossed to C57BL/6J strain for three additional generations [the *cry1*^{-/-}; *cry2*^{-/-} mice we received had already been backcrossed six times (1) before arrival at Salk]. *cry1*^{-/-}; *cry2*^{-/-} mice (8- to 16-week-old) were used for various experiments.

All mice were placed inside light isolation chambers with constant temperature, humidity, and background white noise of approximately 50 dB. Mice were light-entrained under 12 h of white light (800 lux) and 12 h of darkness (LD) for 14 days. One group of mice were fed ad libitum, while the second group was subjected to temporally restricted feeding by granting them access to food for 8 h; from 1 h after lights on (ZT1) till 9 h after lights on (ZT9). After 14 days, all mice were released into DD at ZT12. The feeding schedule was continued until the mice were killed. Mice were killed under dim red light by anesthesia followed by cervical dislocation. The eyes were removed before proceeding with the tissue collection under normal illumination. Three to five WT mice were killed every 1 h, three *cry1*^{-/-}; *cry2*^{-/-} mice were killed every 2 h. To minimize the number of *cry1*^{-/-}; *cry2*^{-/-} mice used in the study, male and female *cry1*^{-/-}; *cry2*^{-/-} mice were killed at alternating time points ("ad libitum" and "restricted feeding" in Fig. S2; male mice were killed at CT 0, 4, 8, 12, 16, 20, and female mice were killed at CT 2, 6, 10, 14, 18, 22).

For assessing temporal expression profile in fasted mice, male C57/B6 mice were given ad libitum access to food for 14 days under LD cycle and released into DD. Food was withdrawn at CT4 or CT16 on the first day in DD, and mice were killed as described above starting at CT2 or CT12, respectively ("Fast-ing-1 and -2" in Fig. S2).

For finding acute effects of feeding on hepatic transcripts, male C57/B6 mice were entrained under LD cycle, held under ad lib condition, and released into DD as described above. On the first day of DD, food was withdrawn for 24 h starting at CT4 (where CT0 corresponds to the time of prior lights on during LD condition). After 24 h of fasting, one group of mice was granted access to food. Fed mice were killed as described above at 2, 4, and 6 h after feeding onset ("Fasting/Re-feeding" in Fig. S2). Mice that did not receive food (earlier paragraph) at comparable time points were used as fasted controls.

Livers were collected and individually frozen in liquid nitrogen. RNA was extracted using RNeasy columns (Qiagen). RNA integrity and concentration were checked by capillary electrophoresis (Experion; Bio-Rad Laboratories).

The RER of individually housed mice were measured by indirect calorimetry in a CLAMS system (Columbus Instruments) at 25 °C. Food intake was measured by continuously measuring the weight of the food supplied for individual animals. Overall food intake was measured by determining the weight of consumed food in bins of 1 week. All mice were fed the normal laboratory diet, Animal Diet #5001 from Lab Diet. Calories came from protein (28.5%), fat (13.5%), and carbohydrates (58%).

Real-Time Quantitative PCR. RNA was isolated with the use of RNeasy Mini kit (Qiagen) according to the manufacturer's

instruction. Equal quantity of total RNA from at least three mice per time point were pooled and used for cDNA synthesis with SuperScript III (Invitrogen). Real-time qPCR was then performed with ABI SYBR Green reagents (Applied Biosystems) following the manufacturer's instructions.

The following primer sequences were used for the indicated genes:

Bmal1: f- ctc gac acg caa tag atg gga, r- ctt cct tgg tcc acg ggt t
Per1: f- tga agc aag acc ggg aga g, r- cac aca cgc cat cac atc aa
Per2: f- gaa agc tgt cac cac cat aga a, r- aac teg cac ttc ctt ttc
Actin: f- ggc tgt att ccc ctc cat cg, r- ca gtt ggt aac aat gcc atg t
Gapdh: f- gac ctc aac tac atg gtc tac a, r- act cca cga cat act cag
Dbp: f- cct gag gaa cag aag gat ga, r- atc tgg ttc tcc ttg agt ctt
ctt g

Temperature Parameters Were Chosen as Follows. At 95 °C for 10 min followed by 40 cycles of 60 °C for 1 min and 95 °C for 15 s. Results for the respective gene of interest (GOI) were normalized to *Actin* or *Gapdh* using the delta^{Ct} method. Namely, the formula $2^{[Ct(\text{control}) - Ct(\text{GOI})]}$ yielded expression values for single time points that were subsequently median normalized to 1 for every time course.

Western Blot Analysis. Proteins were extracted from pulverized frozen liver samples with a lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 8.0, 170 mM NaCl, 50 mM NaF) supplemented with protease- (pepstatin A, aprotinin, leupeptin, soybean trypsin inhibitor; all from Sigma-Aldrich) and phosphatase inhibitors (Phosphatase Inhibitor Mixture I; Sigma-Aldrich), or for blots of pAKT, the samples were extracted with the buffer suggested by the manufacturer of the corresponding antibodies (Cell Signaling). Samples were homogenized in lysis buffer using a Polytron homogenizer (Kinematica). Total liver protein (30 µg) was separated on 10% Tris-glycine acrylamide gels and wet-transferred to PVDF membranes. Antibodies against pCREB (2), CREB (3), pAKT (#7100; Cell Signaling), AKT (#9272; Cell Signaling), and bACTIN (A2066; Sigma-Aldrich) were used at the dilution proposed by the manufacturer in 5% BSA in TBST.

Probe Hybridization and Data Analysis. For each time point, 5 µg total RNA pooled from three individual liver samples was used for reverse transcription and labeled using a One-Cycle Amplification kit (Affymetrix) following the manufacturer's protocol. The resulting labeled cRNA (15 µg) for each time point was fragmented and hybridized to Affymetrix MOE 430.2 HDAs. Separate amplifications and hybridizations were run for samples from WT mice under ad libitum conditions, tRF conditions, and fasting/re-feeding paradigm. Samples from *cry1*^{-/-}; *cry2*^{-/-} under ad libitum and tRF conditions were amplified and hybridized in a single batch. Raw data were analyzed through the gcRMA-algorithm using the Affymetrix package in R (4). Median intensity of all arrays in separate experiments was normalized to 100. Probe sets with median intensities >100 were scored as present. Due to poor array-performance data from CT29 of tRF fed WT mice and CT44 of tRF fed *cry1*^{-/-}; *cry2*^{-/-} mice were excluded from subsequent analysis. Rhythmic transcripts were identified using COSOPT and Fisher G test on expression timelines of ad lib and tRF fed WT and *cry1*^{-/-}; *cry2*^{-/-} mice as described in refs. 5 and 6. Cut-off thresholds for COSOPT and

Fisher G test were determined empirically and set to $p\text{MMC-}\beta < 0.001$ and P value < 0.01 , respectively. Food induced or repressed transcripts were identified by an average induction or repression of 1.6-fold between fasted and fed samples. Transcripts that maintained their rhythmic transcription under fasting conditions were identified by a $p\text{MMC-}\beta < 0.01$ cut-off threshold.

Probe sets were annotated using the Affymetrix annotation file as of September 2007. Heatmaps were generated with the programs Cluster and Treeview (7). The expression data of individual datasets was normalized to a magnitude of 1, median centered, and clustered through average linkage cluster in Cluster. The resulting data were then visualized using Treeview.

1. Gauger MA, Sancar A (2005) Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Cancer Res* 65:6828–6834.
2. Hagiwara M, et al. (1993) Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol Cell Biol* 13:4852–4859.
3. Gonzalez GA, Menzel P, Leonard J, Fischer WH, Montminy MR (1991) Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol Cell Biol* 11:1306–1312.
4. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy—Analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 20:307–315.
5. Panda S, et al. (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109:307–320.
6. Hughes M, et al. (2007) High-resolution time course analysis of gene expression from pituitary. *Cold Spring Harb Symp Quant Biol* 72:381–386.
7. Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95:14863–14868.
8. Siepka SM, Takahashi JS (2005) Methods to record circadian rhythm wheel running activity in mice. *Methods Enzymol* 393:230–239.
9. Kornmann B, Schaad O, Bujard H, Takahashi JS, Schibler U (2007) System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol* 5:e34.

Sample collection outline

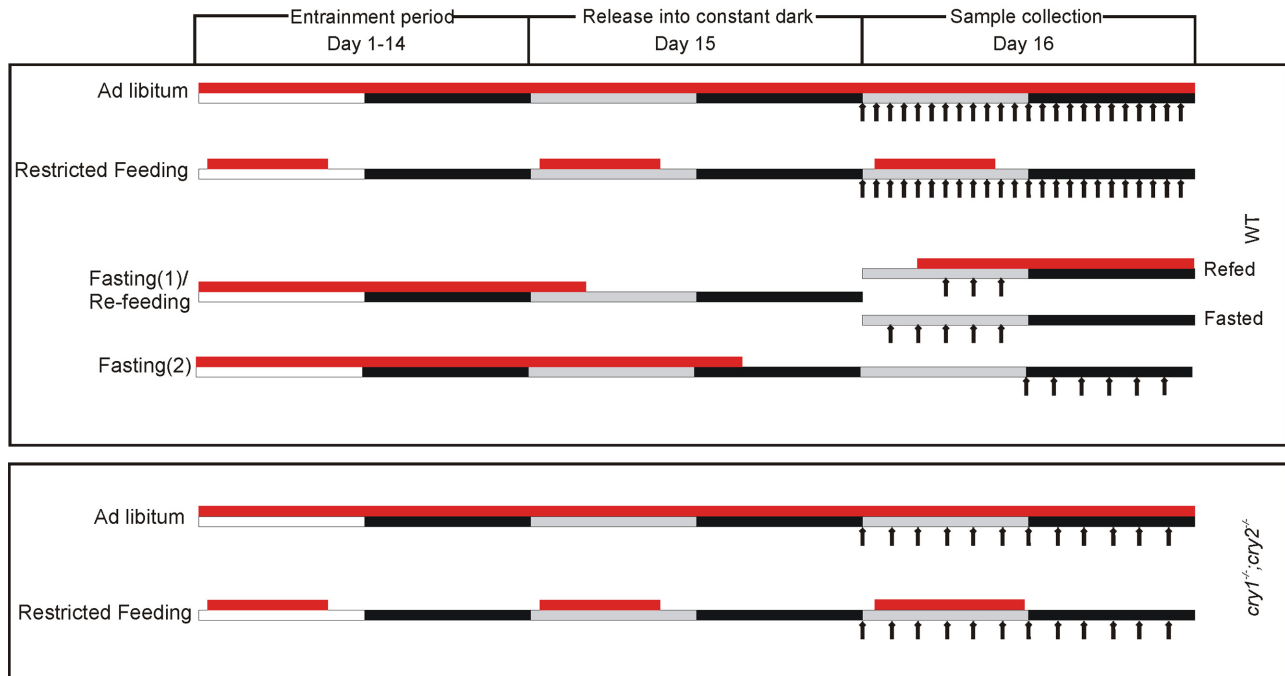
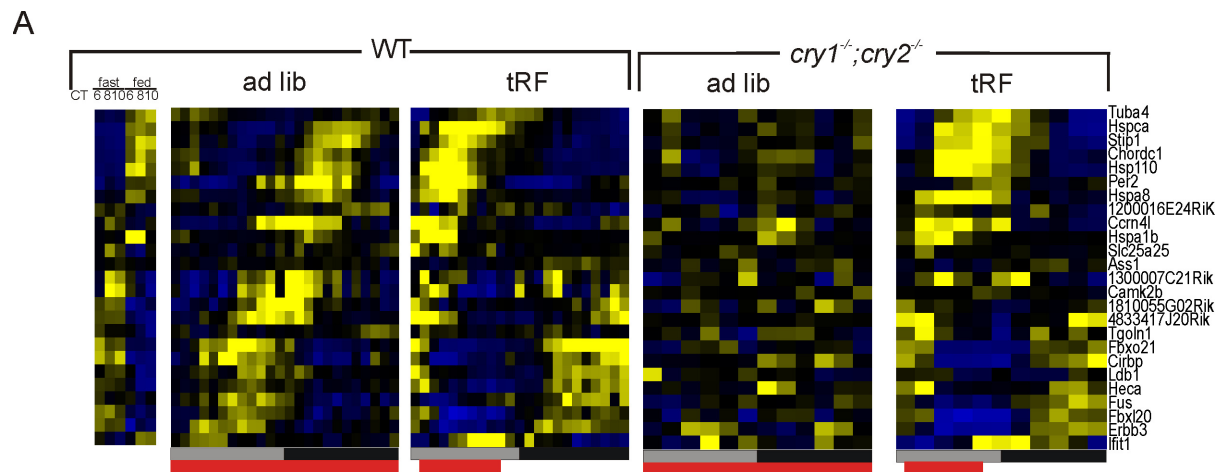


Fig. S2. Experimental outline for HDA and RT-qPCR experiments. WT and *cry1^{-/-};cry2^{-/-}* mice were held under different feeding conditions, and after 2 weeks released into DD. Animals were killed, and livers were collected at time points indicated with black arrows. White bars indicate daytime. Black bars indicate nighttime or subjective nighttime. Gray bars indicate subjective daytime. Red bars indicate food availability.



Rhythmic transcripts in liver-specific *Bmal1* Knock-down mice

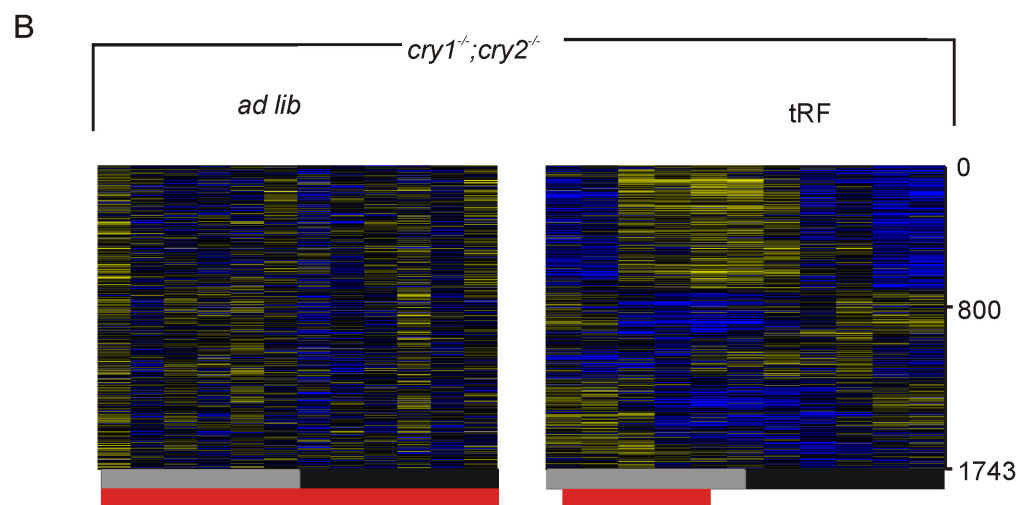


Fig. S3. Feeding consolidation can drive rhythmic transcription. (A) Heatmap rendering of temporal gene expression pattern in the indicated genotypes and feeding conditions of the transcripts, which also show rhythmic transcription in liver-specific *Bmal1* knockdown mice (9) and are considered to be driven by systemic rhythmic cues generated directly or indirectly by the SCN. These transcripts show no rhythmic expression in *Cry*-deficient mice. However, they are modulated by feeding and fasting. Under both ad lib and tRF conditions in WT mice and tRF conditions in *Cry* deficient mice, their expression cycles with peak phases reflecting their response to feeding or fasting. (B) Heatmap rendering of temporal expression patterns of "common cyler" transcripts in *Cry* deficient mice. Transcriptional patterns under tRF conditions show low amplitude rhythms with peak phases similar to WT mice under tRF conditions.

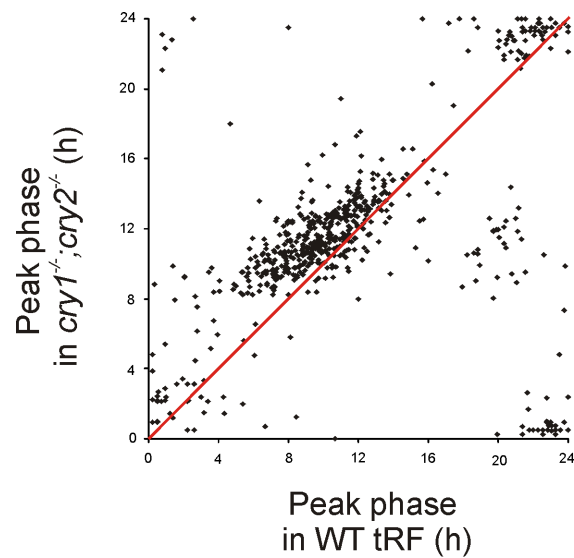


Fig. S6. *Cry1^{-/-};cry2^{-/-}* mice show delayed gene expression. The peak phases of 617 transcripts scored rhythmic in *cry1^{-/-};cry2^{-/-}* mice under tRF were used. The COSOPT-predicted peak phases for WT (x-axis) and *cry1^{-/-};cry2^{-/-}* mice (y-axis) under tRF condition are shown in a scatter plot. The majority of transcripts peak later in *cry1^{-/-};cry2^{-/-}* mice than in WT mice.



Fig. S7. Circadian clock and feeding input cause rhythmic expression of transcripts encoding for mitochondrial proteins. The expression patterns of transcripts encoding for mitochondrial protein as determined by Affymetrix HDAs, which were scored rhythmic in at least one feeding condition were visualized with a heatmap. Different feeding conditions and genotypes are displayed as indicated. Transcriptional rhythms are most robust in tRF of WT mice as compared to mice lacking consolidated feeding or a functional clock.

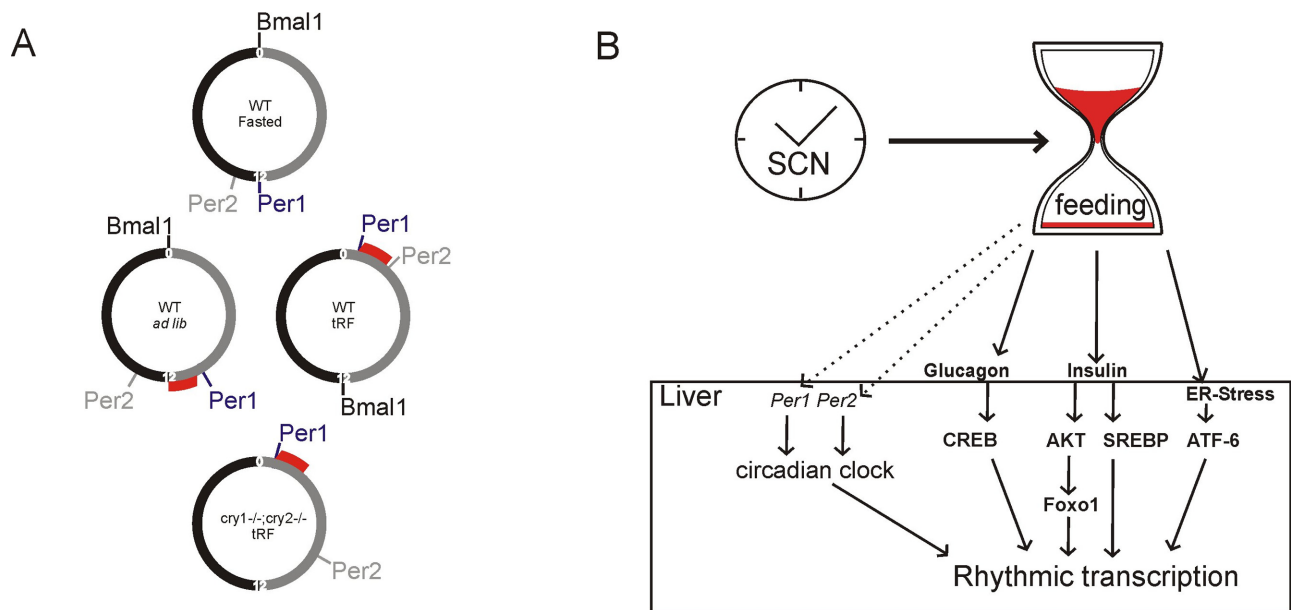


Fig. S8. Feeding impacts rhythmic transcription and the circadian clock. (A) Feeding onset separates the peak expression phases of Per1 and Per2. (B) Feeding induces metabolic regulators, which in turn drive rhythmic transcription in addition to the transcriptional output of the hepatic circadian clock.

	<i>ad lib</i>	tRF	resolution
Wild-type	2,997	4,960	1h
	1,018	1,643	2h
<i>cry1</i> ^{-/-} ; <i>cry2</i> ^{-/-}	16	617	
randomized <i>cry1</i> ^{-/-} ; <i>cry2</i> ^{-/-}	20 ± 15		

Number of rhythmic transcripts (COSOPT MMC- β p value < 0.001 , and Fisher's G test $P < 0.01$) under the indicated condition and sampling frequency are shown. Lower sampling frequency causes lower numbers of identified rhythmic transcripts. Under ad lib conditions *cry1^{-/-};cry2^{-/-}* mice the number rhythmic transcripts is similar to when the expression data is randomized.